Structure of a Penicillin Binding Protein Complexed with a Cephalosporin-Peptidoglycan Mimic

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Many bacteria that are responsible for causing diseases in humans are rapidly developing mutant strains resistant to existing antibiotics. Proliferation of these mutant strains is likely to be a serious health-care problem of increasing proportions. Almost sixty percent of all clinically used antibiotics are beta-lactams, which stop the growth of bacteria by interfering with their cell-wall biosynthesis. The cell walls are made of glycan chains that polymerize to form a structure that gives strength and shape to the bacteria. An understanding of the process how the cell wall is synthesized may help in designing more potent antibiotics.

D-Ala-D-Ala-carboxypeptidase/transpeptidases (DD-peptidases) are penicillin-binding proteins (PBPs), the targets of ß-lactam antibiotics such as penicillins and cephalosporins (Figure 1A). These enzymes catalyze the final cross-linking step of bacterial cell wall biosynthesis. *In vivo* inhibition of PBPs by ß-lactams results in the cessation of bacterial growth.

The bacterial cell wall is primarily composed of peptidoglycan, which forms a sacculus around the inner cell membrane. Peptidoglycan is made up of a polymer of -N-acetylmuramic acid-N-acetylglucosamine- (-NAM-NAG-) repeating saccharide units cross-linked by peptides, typically five residues or more in length. Tipper and Strominger first proposed the hypothesis that ß-lactam antibiotics are effective because they mimic the terminal D-Ala-D-Ala portion of the donor peptidoglycan strand during cross-linking [1].

ß-Lactam antibiotics constitute over 60% of the clinically valuable antibiotics today. We are in danger of losing this important class of antibiotics due to the rapid emergence and spread of drug resistant bacterial strains. Resistance is mostly acquired through the transfer of plasmids encoding ß-lactamases, enzymes that quickly hydrolyze ß-lactam antibiotics rendering them ineffective.

There is strong evidence that ß-lactamases are evolutionary descendents of penicillin binding proteins [2, 3]. It is important then to define why penicillin-binding proteins turn over natural peptidoglycan substrates so rapidly but only slowly deacylate and release ß-lactams. Also, one questions why ß-lactamases hydrolyze ß-lactams and release them rapidly but they do not react with peptide substrates.

To help answer these questions, structural studies of the *Streptomyces* sp. R61 D-Ala-D-Ala-peptidase have been undertaken to investigate how the enzyme

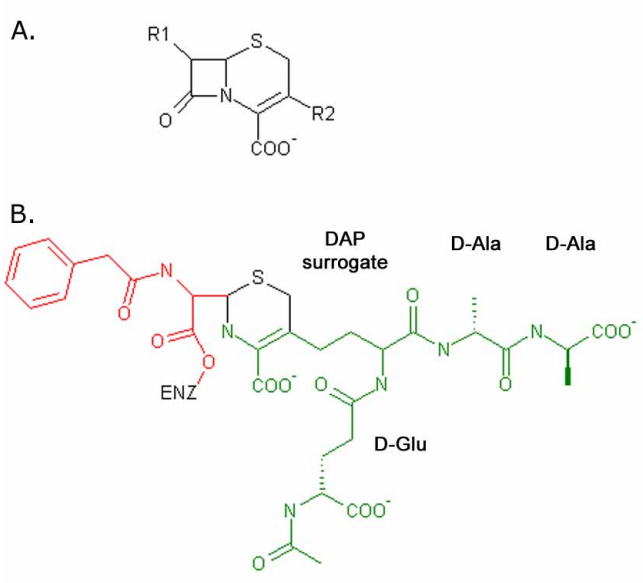

Figure 1. *(A) The cephalosporin nucleus. The highly strained four-membered ß-lactam ring readily reacts with the catalytic nucleophile, usually a serine, of PBPs forming a long-lived acyl-enzyme complex, thus inhibiting the enzyme. (B) Schematic of cephalosporin **1** after formation of the acyl-enzyme complex with the DD-peptidase. The red portion of cephalosporin **1** mimics the D-Ala-D-Ala part of the donor peptide strand, which in the cross-linking reaction is the first substrate attacked by the catalytic serine. The terminal D-Alanine is lost and an acyl-enzyme is formed with the penultimate D-Alanine. The green portion of Cephalosporin **1** mimics the second substrate, or acceptor strand, poised for attack on the carbonyl carbon of the acyl-enzyme to complete cross-link formation between the donor and acceptor strands.*

binds to and reacts with a peptidoglycan analog. This enzyme has been used extensively as a model for membrane bound PBPs [4, 5]. Since previous structural studies using di- and tri-peptide substrates have been unsuccessful in trapping a PBP enzyme-substrate complex [2, 6], a new approach described here was developed.

A novel cephalosporin, called cephalosporin **1**, was designed and synthesized with side chains that mimic peptidoglycan strands typically found in bacteria (Figure 1B). Since a long-lived, covalent complex is formed with cephalosporin **1**, it has proven useful for capturing crystallographically an image of this enzyme-substrate analog complex [7].

Crystals of the R61 DD-peptidase were grown as previously described [6]. Pre-formed DD-peptidase crystals were soaked in a 5.4mM solution of cephalosporin **1** for four days to allow enough time for the compound to react. Following the soak, X-ray data were collected at NSLS Beamline X12C on the acyl-enzyme intermediate [7].

Data sets for protein structures with resolutions better than 1.2Å and I/sig<I> greater than 2 in the last resolution bin are considered to be ultra-high resolution data [8]. With the use of synchrotron radiation sources, ultra-high resolution data are becoming more common in the structures deposited in the RCSB-PDB. Other factors enhancing protein crystallographic studies today are the development of CCD detectors with large areas and fast image readout times, and the development of cryo-techniques [9].

Crystals of the DD-peptidase/cephalosporin **1** complex diffracted to ultra-high resolution, 1.2 Å. This resolution permitted the atomic coordinates to be determined with great accuracy and also allowed the assignment of 10% of possible protein hydrogen atoms based on difference electron density maps. Initial phases were based on the wild type 1.6Å structure [10]. Refinement was carried out initially in CNS and continued in SHELXL, which is more suited to refinement of anisotropic displacement parameters [11, 12] (See Tables 1 and 2).

The resulting structure gives, for the first time, a snapshot of how the natural peptidoglycan would bind to and react with PBPs. The complex reveals deep grooves on the surface of the enzyme that accommodate binding of the donor and acceptor peptidoglycan strands, the substrates in the natural reaction. The

Table 1

Crystallographic Data	
Resolution limit	1.2 Å
Number of reflections	
Measured	2,333,719
Unique	111,373
Reflections, F > 4 σ	93,339
Completeness	
All data	96 %
Highest resolution shell (1.21-1.17 Å)	73 %
R sym* (on I)	0.040
< I / Sigma (I) > overall	25.0
Refinement	
Resolution	∞ - 1.17Å
R factor (no cutoff)	0.115
R free (no cutoff)	0.154
Data in test set	5,560
Non-hydrogen atoms	3,337
Rms	
Bond lengths (Å)	0.015
Angle distances (Å)	0.030
Average B of ligand	33.3Å^2

* R sym = Σ | Iobs - < I > | / Σ < I >

Table 2
SHELXL refinement of R61 - Cephalsporin 1 complex

Job	Action taken	NP	NH	NW/NW½	N_{par}	R_1	R_{free}
1	Final X-PLOR, 1.17-8Å		2610	0	441 / 0	22.57	24.55
2	SHELXL, no alternate conformations		2610	0	441 / 0	12204 18.67	21.03
3	SWAT added		2656	0	478 / 0	12536 18.20	20.70
4	All atoms anisotropic		2656	0	478 / 0	27462 13.70	17.21
5	Alternate conf., ligand and solvent		2727	0	504 / 0	28353 12.82	16.60
6	More alternate conf. and solvent		2712	0	524 / 0	28695 12.74	16.39
7	More alternate conf. and solvent		2717	0	536 / 0	29112 12.23	15.89
8	More alternate conf. and solvent		2721	0	556 / 0	29274 12.04	15.74
9	More alternate conf. and solvent		2760	0	562 / 0	30068 11.47	15.38
10	Half occupancy waters		2760	0	511 / 30	29825 11.58	15.40
12	10% of total hydrogens added		3052	276	511 / 30	29744 11.45	15.34
13	All data		3035	276	521 / 39	29987 11.39	

NP: number of protein atoms; NH: number of hydrogen atoms; NW/NW½:number of waters/half occupancy waters; N_{par}: number of parameters; R_1 and R_{free}: crystallographic and free residuals.

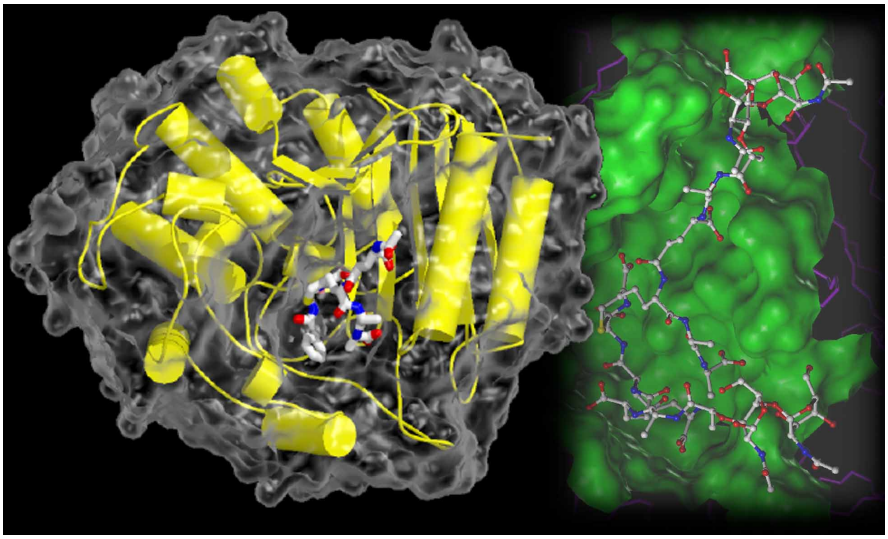

Figure 2. *(Left) DD-peptidase secondary structure elements represented as cylinders for helices, ribbons for beta-strands, and coil for loop regions, all in yellow. A transparent surface of the enzyme helps to convey the depth of the binding grooves while still allowing a view of the overall structure. The bound Cephalosporin* ***1*** *molecule is shown as sticks with the CPK coloring scheme. (Right) The binding grooves for peptidoglycan strands are clearly seen in the green enzyme surface representation. NAM-NAG residues have been modeled on to the crystal structure of the acyl enzyme, fitting the disaccharides into the obvious grooves on the surface of the enzyme. The model was then energy minimized.*

grooves position the strands in the active site so that the cross-linking reaction could take place (Figure 2 - left).

Modeling of a NAM-NAG dimer onto each peptide fragment of the crystal structure indicates additional grooves on the enzyme surface that would accommodate the polysaccharide portion of peptidoglycan. This extended structure was energy minimized without significant change in position, signifying that the model is realistic [7] (Figure 2-right). This new knowledge of PBP substrate binding sites will aid in the development of more effective antibiotics.

References

[1] Tipper, D.J. and J.L. Strominger, *Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine.* Proc. Natl. Acad. Sci. U.S.A., 1965. **54**: p. 1133-1141.

[2] Kelly, J.A., *et al.*, *Penicillin target enzyme and the antibiotic binding site.* Science, 1982. **218**: p. 479-481.

[3] Massova, I. and S. Mobashery, *Kinship and diversification of bacterial penicillin-binding proteins and ß-lactamases.* Antimicrob. Agents Chemother., 1998. **42**: p. 1-17.

[4] Frere, J.M. and B. Joris, *Penicillin-sensitive enzymes in peptidoglycan biosynthesis.* CRC Crit. Rev. Microbiol., 1985. **11**: p. 299-396.

[5] Kelly, J.A., *et al.*, *2.8 Å structure of penicillin-sensitive DD-peptidase from Streptomyces R61 and complexes with ß-lactams.* J. Biol. Chem., 1985. **260**: p. 6449-6458.

[6] Kelly, J.A., *et al.*, *Crystallographic mapping of ß-lactams bound to a DD-peptidase.* J. Mol. Biol., 1989. **209**: p. 281-295.

[7] Lee, W., *et al.*, *A 1.2-A snapshot of the final step of bacterial cell wall biosynthesis.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1427-1431.

[8] Sheldrick, G.M., *Phase annealing in SHELX-90: direct methods for larger structures.* Acta Crystallogr A, 1990. **46**: p. 467-473.

[9] Dauter, Z., V.S. Lamzin, and K.S. Wilson, *The benefits of atomic resolution.* Curr Opin Struct Biol, 1997. **7**(5): p. 681-8.

[10] Kelly, J.A. and A.P. Kuzin, *Refined crystallographic structure of a DD-peptidase penicillin target enzyme at 1.6 Å resolution.* J. Mol. Biol., 1995. **254**: p. 223-236.

[11] Brunger, A.T., *et al.*, *CNS (Crystallography and NMR System).* Acta Crystallogr., 1998. **D54**: p. 905-921.

[12] Sheldrick, G.M. and T.R. Schneider, *SHELXL: high resolution refinement.* Methods in Enzymology, 1997. **277**: p. 319-343.